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THYROTROPIN-RELEASING HORMONE STIMULATES A CALCIUM-ACTIVATED POTASSIUM CURRENT IN A RAT ANTERIOR PITUITARY CELL LINE

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SUMMARY

1. The 'giga-seal' patch-electrode technique (Hamill, Marty, Neher, Sakmann & Sigworth, 1981) was used for constant current and voltage-clamp recordings in the GH3 rat anterior pituitary cell line.

2. Thyrotropin-releasing hormone (TRH) causes a membrane hyperpolarization that is mediated by a selective increase in K^+ permeability. The hyperpolarization cannot be evoked when the cell is internally perfused with a Ca^{2+} chelator but persists when the external solution that bathes the cell is Ca^{2+}-free or contains a Ca^{2+}-channel blocker.

3. Under voltage clamp the TRH-induced current is approximately linear at negative potentials (−90 to −30 mV) but markedly enhanced at voltages above −30 mV). Thus, the affected conductance has a voltage-dependent component.

4. The TRH-induced increase in K^+ permeability is sensitive to inhibition by 30 mM-TEA and 200 nM-apamin, inhibitors of two distinct Ca^{2+}-activated K^+ permeabilities in GH3 cells.

5. The time course of the TRH-induced K^+ current is similar to the time course of a TRH-induced transient peak elevation of cytosolic Ca^{2+} that is due to mobilization of Ca^{2+} from intracellular stores.

6. The effects of TRH on the K^+ current and the rise in cytosolic Ca^{2+} are half-maximal at 7 nM and 1·7 nM, respectively.

7. It is concluded that the TRH-induced hyperpolarization is mediated by two distinct Ca^{2+}-activated K^+ conductances that are activated by release of Ca^{2+} from an intracellular site.

INTRODUCTION

The GH3 cell line was cloned from a rat anterior pituitary tumour (Tashjian, Yasamura, Levine, Sato & Parker, 1968) and has been shown to secrete prolactin and growth hormone via a Ca^{2+}-dependent mechanism (Tashjian, Lomedico & Maina, 1978; Gautvik, Iversen & Sand, 1980). Secretion of prolactin from GH3 cells is stimulated by thyrotropin-releasing hormone (TRH), a tripeptide of hypothalamic origin which also stimulates prolactin secretion from the anterior pituitary gland

(Hinkle & Tashjian, 1973; Dannies & Tashjian, 1976). TRH also affects the membrane electrical properties of GH3 cells (Kidokoro, 1975; Ozawa & Kimura, 1979; Dufy, Vincent, Fluery, Dupasquier, Gourdji & Tixier-Vidal, 1979; Ozawa, 1981). TRH causes a transient early hyperpolarization which lasts for 90 s or less. This is followed by a long-lasting decrease in membrane conductance and coincident depolarization with facilitation of Ca^{2+}-dependent action potentials. During the long-lasting phase TRH causes silent cells to fire spontaneously and spontaneously firing cells to fire at a higher frequency. In this report, the transient early hyperpolarization, which appears to be mediated by a Ca^{2+}-activated K^+ conductance (Ritchie, 1983; Dubinsky & Oxford, 1985), is examined in further detail. The activation is shown to occur secondarily to the release of Ca^{2+} from an intracellular site and to involve two distinct types of Ca^{2+}-activated K^+ conductances. The relevance of these results is discussed in relation to the TRH-induced stimulation of prolactin secretion. A preliminary account of this work has appeared elsewhere (Ritchie, 1983).

METHODS

Cells. GH3 cells were grown in a 5 % CO_2 atmosphere at 37 °C in HAM's F10 medium containing 15 % (v/v) heat-inactivated horse serum and 2·5 % (v/v) fetal calf serum. For electrical recordings cells were grown in 35 mm tissue culture dishes. Cells that were used for measurements of intracellular Ca^{2+} levels were grown for 1 or 2 days in plastic Petri dishes in a suspension medium (Spinner) containing 2·5 % fetal calf serum and 15 % horse serum.

A few of the current-clamp results were obtained from GH3 cells that had been treated with polyethyleneglycol (PEG; MW 1000, 50 % w/v in HAM's F10 medium, pH 7·4) for 1 min (O'Lague & Huttner, 1980). This procedure caused cell fusion and thereby produced large multinucleated cells (up to 50 μm in diameter) that were particularly helpful for obtaining long-term stable recordings under constant-current conditions. No qualitative differences, other than improvement in membrane potential (usually −40 to −50 mV), were observed in PEG-treated *vs.* untreated cells. All of the voltage-clamp data were obtained from cells that were not treated with PEG and that had diameters between 12 and 24 μm.

Recording. The 'giga-seal' patch-electrode technique (Hamill *et al.* 1981) was used for whole-cell recording. Detailed descriptions of the methodology and the voltage-clamp recording configuration are in the preceding paper (Ritchie, 1987). For recording under constant current, the patch electrode was connected to a KS700 high-impedance ($10^{11}\,\Omega$) unity-gain amplifier (W-P Instruments, Inc., U.S.A.). Current was injected into the cell through the recording electrode and nulled via a bridge balance circuit. In consideration of the high input resistance of the cells (0·5–5 GΩ), the gate leakage current was carefully adjusted at daily intervals. All recordings were performed at room temperature.

Solutions. The composition of 'normal' saline solution was 3 μM-TTX, 150 mM-NaCl, 5·6 mM-KCl, 2 mM-$CaCl_2$, 1 mM-$MgCl_2$, 10 mM-glucose and 10 mM-HEPES, buffered to pH 7·3 with NaOH. In a few instances, a low-Cl^- saline solution was prepared by using the gluconate salt of Na^+ and K^+. Changes in the composition of solution bathing the cell during electrical recording were accomplished by pressure ejection of solution on to the cell from a large-bore pipette (for details, see Ritchie, 1987).

The intracellular patch electrode contained 150 mM-KCl or potassium gluconate in addition to 3 mM-HEPES. In some instances 20 mM (*trans*-1,2-cyclohexylenedinitrilo) tetraacetic acid (CDTA) was also present and an osmotic equivalent of KCl or potassium gluconate was removed. The pH was adjusted to 7·3 with KOH. The pipette resistances were 3–8 MΩ.

The liquid-junction potential between the internal (patch electrode) and the external (bath) solutions were measured (see Ritchie, 1987). The values determined, internal relative to external, were the following: 150 mM internal KCl *vs.* normal saline soution, −3·5 mV; 150 mM internal potassium gluconate *vs.* normal saline solution, −12 mV; 150 mM internal potassium gluconate *vs.*

low-Cl^-, gluconate-substituted saline solution, −3·5 mV. Except when specifically stated, the data presented are not corrected for the liquid-junction potential.

Measurements of intracellular Ca^{2+}. Intracellular Ca^{2+} levels were monitored by measuring the fluorescence of quin-2 (Tsien, Pozzan & Rink, 1982). The cells (5×10^6 cells) were incubated in 1 ml of a HEPES-buffered HAM's F10 medium containing 20 μM-quin-2/AM, the acetoxymethyl ester of quin-2 (quin-2/AM was diluted from a 4 mM stock solution in dimethyl sulphoxide). After a 20 min incubation at 37 °C the cells were diluted with 10 ml of saline solution and incubated for an additional 20 min. The cells were centrifuged, washed with 10 ml of saline solution then placed in 1 ml of test saline solution and transferred to a quartz cuvette. The cells were kept in suspension with a magnetic stirrer. Fluorescence was monitored at room temperature on a Perkin Elmer 650-10S spectrofluorometer at excitation and emission wave-lengths of 340 and 498 nm, respectively. The observed emission maximum was 498 nm. The intracellular Ca^{2+} concentration, $[Ca^{2+}]_i$, was determined by using the equation described by Tsien *et al.* (1982): $[Ca^{2+}]_i = (K_D)(F\text{-}F_{min})/ F_{max}\text{-}F)$. The K_D (dissociation constant) for the Ca^{2+}–quin-2 complex at room temperature is 80 nM (Tsien, 1980). F is the fluorescence intensity emitted from intact cells. F_{max} is the maximum fluorescence obtained upon disruption of the cells with 0·1% (w/v) Triton X-100. F_{min} is the minimum fluorescence obtained by a subsequent addition which results in a final solution that contains 15 mM-K_2EGTA and 30 mM-Tris with a pH of 8·3. A correction was employed to compensate for a small decrease in blank fluorescence that occurs when Triton is added to cells that are not treated with quin-2/AM. The intracellular quin-2 concentration in these experiments was 114 ± 48 μM (mean ± S.D., $n = 22$), a concentration which does not interfere with TRH stimulation of prolactin release (Gershengorn & Thaw, 1983; Albert & Tashjian, 1984*a*).

RESULTS

Characterization of the TRH-induced conductance

As first shown by Ozawa & Kimura (1979), TRH (50 nM) induces an early hyperpolarization with increased conductance (Fig. 1). This hyperpolarization lasts between 20 and 90 s. Ozawa & Kimura (1979) also observed a later depolarization with decreased conductance and coincident facilitation of action potential frequency. In the present study this late response was rarely observed. Thus, only the early hyperpolarization is characterized.

Determination of ion selectivity. Due to slow recovery of the transient hyperpolarizing response to TRH it is necessary to wait 6–10 min before application of a successive dose of TRH. Even at these intervals successive responses are often slightly attenuated compared to the previous response. The reversal potential of the TRH response was therefore determined by interpolation of the responses observed on a single application of TRH but simultaneously monitored at two different membrane potentials (Fig. 1). This was accomplished by passing hyperpolarizing current pulses at regular intervals as described by Ozawa & Kimura (1979). Variability was minimized by calculating the mean reversal potential after repeating the test at least twice more in the same cell. The reversal potential, after correction for the liquid-junction potential, that was determined in five different cells was -80 ± 1 mV (mean ± S.E. of mean) in normal saline solution. The relation between reversal potential and K^+ concentration (Fig. 1) was observed to vary by 58 mV/decade change in external K^+. This is identical to the theoretical slope predicted for a selective increase in K^+ permeability.

The response to TRH was also examined under voltage clamp. Application of 50 nM-TRH elicited an outward current at −50 mV and an inward current at −90 mV (Fig. 2). The reversal potential, after correction for the liquid-junction potential, was

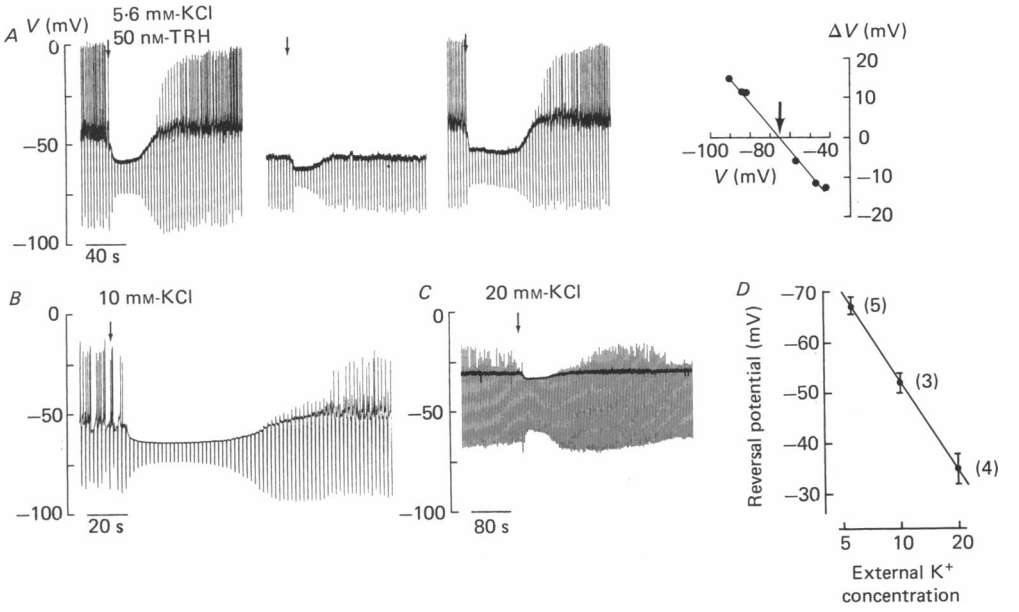

Fig. 1. Reversal potential of the early hyperpolarization in response to 50 nM-TRH. *A*, the membrane response to a 1 s application of 50 nM-TRH (applied at the arrow) was measured while passing 400 ms hyperpolarizing current pulses. This allowed the TRH response to be sampled simultaneously at two different membrane potentials. 6–10 min later, a second application of TRH was applied while the membrane was hyperpolarized to a different level with d.c. current. This process was then repeated a third time at two more membrane potentials. The amplitude of the voltage response (ΔV) during maximal increase in conductance is plotted as a function of membrane potential on the right. This graph represents a rare instance in which the membrane responses to multiple applications of TRH could be fitted by one straight line suggesting that the conductance change was identical in these successive applications. The reversal potential (indicated by the arrow) is the membrane potential which gives rise to no response. It is −67 mV in this example (or −79 mV after correction for the liquid-junction potential). The second row shows responses in 10 (*B*) and 20 (*C*) mM-K^+. *A*, *B* and *C* represent responses obtained from different cells. As the external K^+ concentration is increased the reversal potential shifts to more positive levels. The reversal potential is plotted as a function of the log of external K^+ concentration in *D*. Each point represents the mean ± S.D. with the number of cells examined in parentheses. The reversal potential for each cell was determined from the mean of at least three different applications of TRH to the same cell. These results were obtained from cells that were treated with PEG. The intracellular electrode contained 150 mM-potassium gluconate and 3 mM-HEPES.

-76 ± 1 mV (mean ± S.E. of mean, $n = 5$). This is similar to the value obtained under constant-current recording. Measurements included in the average were obtained from cells that were bathed in normal saline or in low-Cl^- saline solution. This confirms that the reversal potential of the TRH-induced current is not affected by changes in Cl^- concentration (Ozawa & Kimura, 1979).

Ca^{2+} dependence of the TRH response. In normal Ca^{2+} saline solution but in the presence of a Ca^{2+}-channel blocker (0·5 mM Cd^{2+}; Fig. 3), TRH continues to evoke a hyperpolarization with increased conductance. The TRH-induced increase in K^+ current also persists in a 0 Ca^{2+}, 0·5 mM-EGTA saline solution (for example, see Fig. 8). Thus, the TRH-induced increase in K^+ permeability does not require extracellular Ca^{2+}.

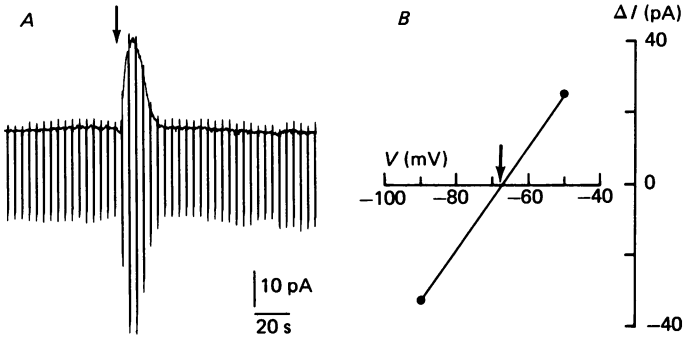

Fig. 2. Measurement of the reversal of the TRH response under voltage clamp. This cell was bathed in a low-Cl^-, gluconate-substituted saline solution. The intracellular electrode contained 150 mM-potassium gluconate and 3 mM-HEPES at pH 7·3. The cell was voltage clamped at −50 mV and stepped to −90 mV for 400 ms every 4 s. The current trace is shown in *A*. TRH (50 nM) was applied at the arrow for 5 s. A plot of the magnitude of the current change (ΔI) at −50 and −90 mV is shown in *B*. The reversal potential, the potential where application of TRH would give rise to no change in net current, is indicated by the arrow (−68 mV in this Figure or −71·5 mV after correction for the liquid-junction potential).

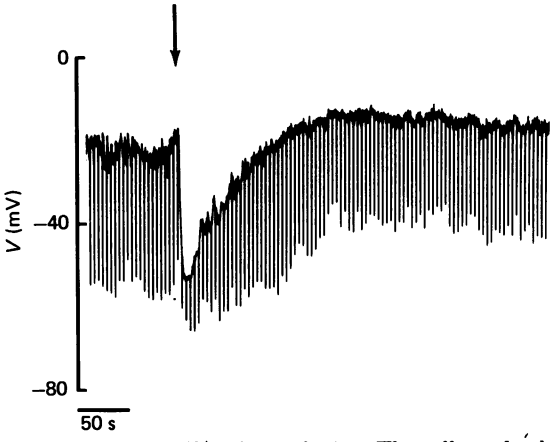

Fig. 3. Effect of TRH in 0·5 mM-Cd^{2+} saline solution. The cell was bathed in saline solution containing 0·5 mM-Cd^{2+} and the recording electrode contained 150 mM-potassium gluconate and 3 mM-HEPES, pH 7·3. Hyperpolarizing pulses were applied at regular intervals to monitor changes in input resistance. TRH (50 nM) was applied at the arrow for 1 s causing a transient hyperpolarization with a concomitant decrease in input resistance. This result was obtained from a PEG-treated cell.

The Ca^{2+} requirement was also examined by placing a Ca^{2+} chelator in the recording pipette. When intracellular Ca^{2+} was chelated by inclusion of 20 mM-CDTA in the recording pipette the durations of the spontaneous action potentials became progressively longer (up to 5 s long) as the CDTA gradually leaked out of the patch pipette and into the cell (Fig. 4*B*). In an earlier study, it was demonstrated that this concentration of CDTA completely abolishes Ca^{2+}-activated K^+ currents in GH3 cells (Ritchie, 1987). TRH was then applied and failed to elicit a response in nine out of nine cells tested. Control cells from matched sets of cultures gave a TRH-induced hyperpolarization in eight out of eight cells tested (Fig. 4*A*). These results indicate

that the TRH-induced K^+ permeability increase is dependent on intracellular Ca^{2+}, but not on extracellular Ca^{2+}. However, an effect of CDTA that is unrelated to chelation of Ca^{2+} cannot be excluded.

Pharmacological inhibition of the TRH-induced K^+ current. The requirement for intracellular Ca^{2+} is consistent with the suggestion that Ca^{2+}-activated K^+ channels

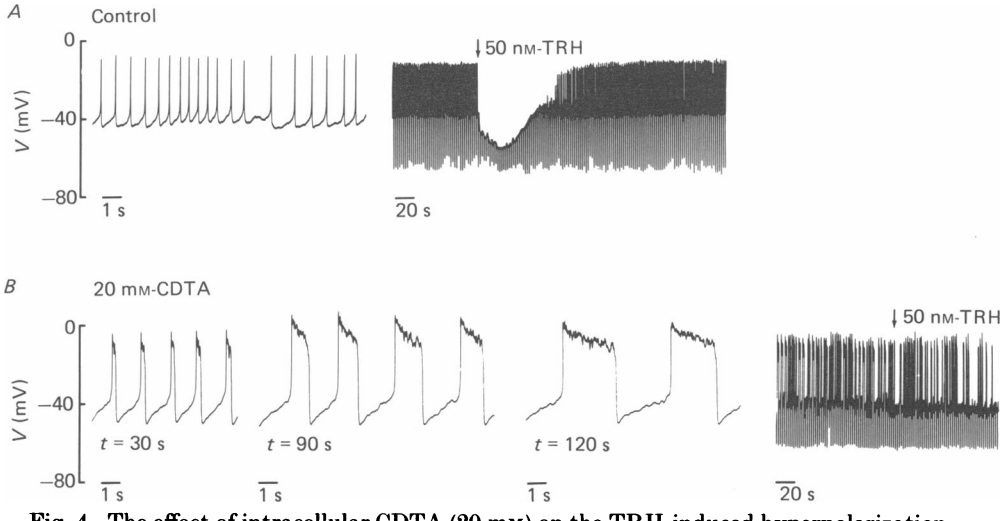

Fig. 4. The effect of intracellular CDTA (20 mM) on the TRH-induced hyperpolarization. The cells were bathed in control saline solution. The recording electrode contained 150 mM-potassium gluconate and 3 mM-HEPES, pH 7·3 in *A* and 120 mM-potassium gluconate, 20 mM-CDTA and 3 mM-HEPES, pH 7·3 in *B*. The shape of the spontaneous action potentials is shown at the left. The response to a 1 s application of TRH (50 nM) is shown at the right. In cell *B*, there is a progressive increase in the duration of the Ca^{2+} action potential as CDTA leaks into the cell from the recording electrode. The amount of elapsed time after introduction of the recording electrode into the cell is indicated immediately below the corresponding voltage traces (*t*). Subsequent application of TRH failed to produce a hyperpolarization in this cell, as well as in eight other cells tested under the same conditions. These results were obtained from cells that were treated with PEG. The diameters of control cells and cells tested with intracellular CDTA were similar (22 ± 2 μm; mean ± S.D.).

are involved in mediating the TRH-induced increase in K^+ permeability (Dubinsky & Oxford, 1985). In the previous article (Ritchie, 1987), the existence of two distinct Ca^{2+}-activated K^+ conductances in GH3 cells was demonstrated. In some cells, 30 mM-TEA inhibits all of the Ca^{2+}-activated K^+ permeability. Most cells, however, contain a second pharmacologically distinct Ca^{2+}-activated K^+ permeability that is relatively resistant to inhibition by TEA but is blocked by 200 nM-apamin. These two pharmacological agents were tested to determine if the TRH-induced K^+ permeability increase could be ascribed to either conductance.

The TRH-induced K^+ current is inhibited to varying degrees by either TEA (30 mM) or apamin (100 nM). Fig. 5 illustrates a TRH-induced K^+ current that was completely and reversibly inhibited by 30 mM-TEA. Some cells, however, still exhibit a response to TRH in the presence of 30 mM-TEA (data not shown). Thus 30 mM-TEA does not completely inhibit the TRH-induced K^+ current in all cells. Fig. 5*B* contains

an example of a TRH response that was markedly attenuated but not abolished by apamin. In some instances (not shown), however, apamin had no effect. Thus, the TRH-induced K^+ current shows the same pharmacological profile as the Ca^{2+}-activated K^+ currents in GH3 cells.

In the example shown in Fig. 5*A*, the TRH-induced outward current is superimposed on a slower-developing inwardly directed change in the holding current that is insensitive to TEA. The time course of the inward change in current has a slow

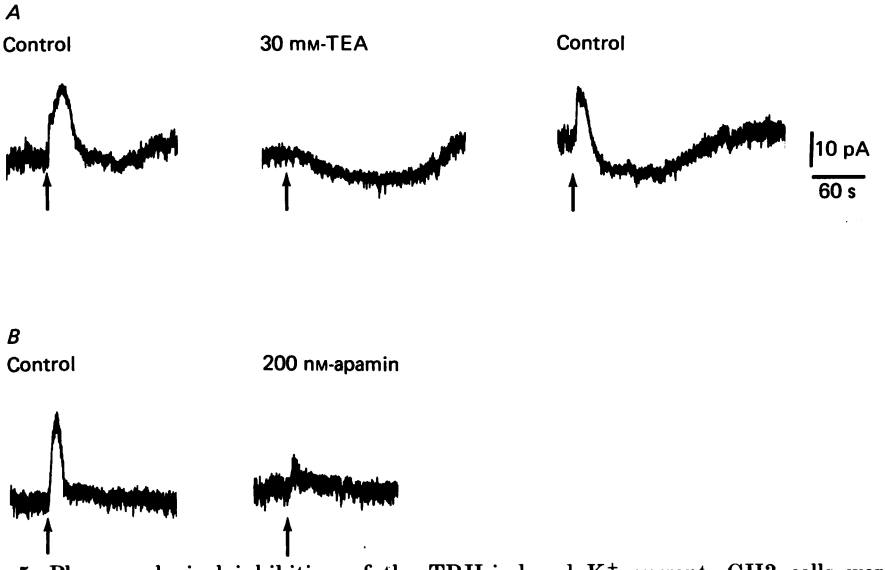

Fig. 5. Pharmacological inhibition of the TRH-induced K^+ current. GH3 cells were voltage clamped to −45 mV. The intracellular electrode contained 150 mM-KCl and 3 mM-HEPES, pH 7·3. TRH (50 nM) was applied to the cell for 5 s in the absence and presence of inhibitors of the Ca^{2+}-activated K^+ currents. *A*, the dish was continuously perfused with normal saline solution or with saline solution containing 30 mM-TEA. At the arrow 50 nM-TRH in normal saline solution or in 30 mM-TEA saline solution was pressure ejected onto the cell. The response in TEA and the subsequent response after wash-out of TEA were elicited 6 and 12 min, respectively, after the first control response. In this cell TEA completely and reversibly inhibited the TRH-induced outward K^+ current but did not interfere with a slower developing, longer-lasting TRH-induced net inward current. *B*, these two responses were elicited 6 min apart and were obtained before and 2 min after addition of 200 nM-apamin to the recording dish. At the arrow 50 nM-TRH was pressure ejected onto the cell.

onset that is similar to a late long-lasting decrease in conductance that is occasionally seen under constant-current recording conditions. This experiment demonstrates that the slowly developing current is not due to TRH-mediated inhibition of the TEA-sensitive Ca^{2+}-activated K^+ permeability. Due to the infrequent occurrence of the slowly developing response the apamin sensitivity was not determined.

Voltage dependence of the K^+ current that is induced by TRH. The Ca^{2+}-activated K^+ conductance in many cells is also voltage sensitive such that for any given level of intracellular Ca^{2+} the opening probability of the channel increases as the membrane is depolarized to more positive potentials (Barrett, Magleby & Pallotta, 1982). It was thus of interest to determine if the TRH-induced Ca^{2+}-activated K^+ current in GH3

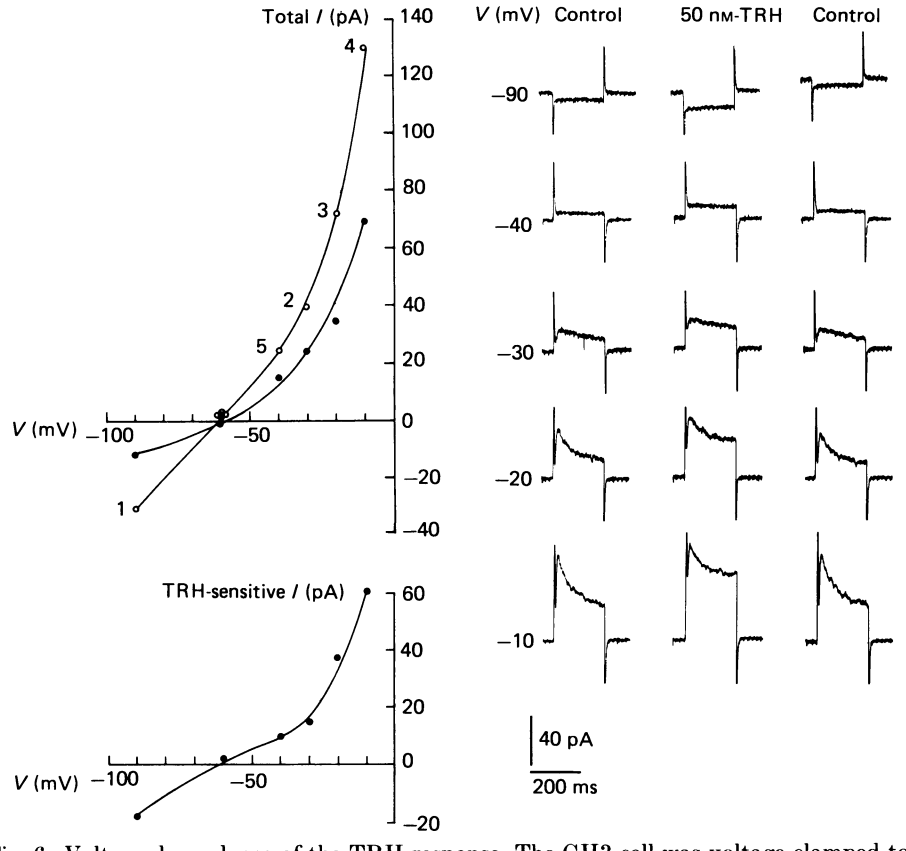

Fig. 6. Voltage dependence of the TRH response. The GH3 cell was voltage clamped to −60 mV. The bath contained normal saline solution and the recording electrode contained 150 mM-potassium gluconate and 3 mM-HEPES, pH 7·3. Control responses were obtained by stepping the cell to a 400 ms test potential every 10 s (first column of current traces). The current response at the end of the 200 ms pulse is plotted in the *I–V* curve to the left (●). The responses to TRH were made by recording the response to a similar voltage step after application of 50 nM-TRH for 1 s. The maximum response to TRH is shown in the middle column of current traces. These results are also plotted in the *I–V* curve to the left (○). The TRH responses at the different voltages were elicited at 6 min intervals. The small numbers adjacent to each open circle indicate the order in which the responses were measured. In each instance the amplitude of the response in the presence of TRH is larger. The reversibility is shown by the return of control responses shown in the final column of current traces. The difference between control and TRH *I-V* curves is plotted in the lower Figure.

cells was also voltage sensitive. In a voltage-clamped cell that showed highly reproducible responses to repeated applications of TRH, TRH was applied five times at 6 min intervals while stepping to a different test potential for each successive application of TRH. The voltage steps were applied for 200 ms at 10 s intervals and the maximal effect of TRH was observed within the first 10 s. The actual current traces are shown at the right in Fig. 6. The current amplitudes at the end of a 200 ms voltage step in control (●) and during TRH (○) are plotted in the current–voltage (*I-V*) graphs to the left. Complete recovery was obtained within 6 min after each application of TRH. The specific TRH-induced current is plotted in the lower graph

as the difference after subtraction of control current. The curve is nearly linear at very negative membrane potentials but increases steeply at potentials above −30 mV. Although this was the only cell in which it was possible to test the response at six different potentials, the responses at three or four different potentials have been tested in numerous cells with similar results. Thus the TRH-induced K^+ current demonstrates a voltage sensitivity at positive potentials which is consistent with

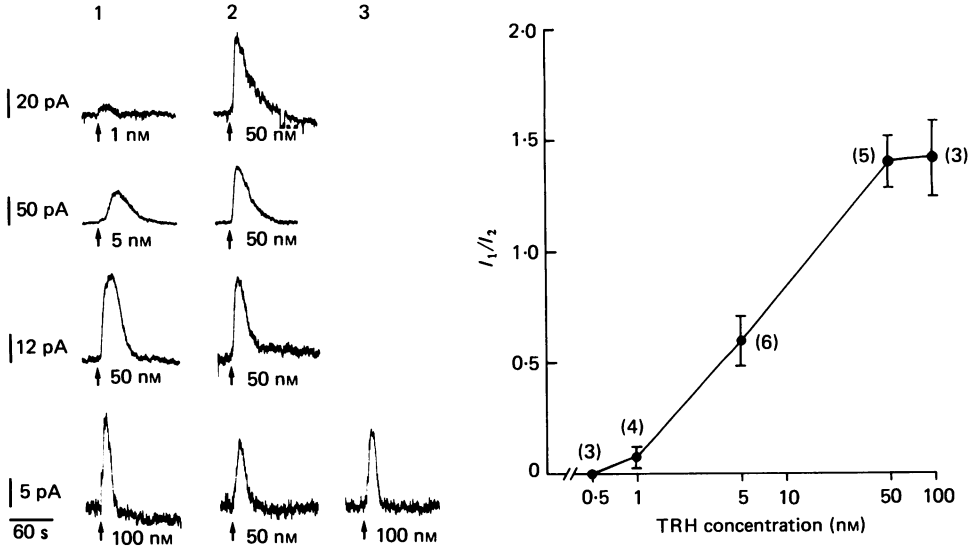

Fig. 7. Dose dependence of the TRH-induced K^+ current. The current traces to the left represent examples from four different cells that were voltage clamped to −45 mV in normal saline solution. The intracellular electrode contained 150 mM-KCl and 3 mM-HEPES, pH 7·3. At the first arrow (column 1) the indicated concentration of TRH was applied for 5 s. At the second arrow (column 2) 50 nM-TRH was applied for 5 s to the same cell 6 min later. The bottom row of traces includes a third response elicited 6 min after response 2. The dose–response curve plotted to the right represents the amplitude of the first response divided by the amplitude of the second response and plotted against the log of the TRH concentration used to elicit the first response. The EC_{50} is approximately 7 nM.

behaviour that is predicted for a response that involves voltage-sensitive Ca^{2+}-activated K^+ channels. The near linearity of the response at negative potentials suggests that the channels that are sensitive to Ca^{2+} at potentials near rest may be different from the Ca^{2+}-activated K^+ channels that are activated at potentials positive to −30 mV.

Dose-response relation of the TRH-induced K^+ current. The dose-response relation of the TRH-induced K^+ current was obtained by using a normalization procedure that helped minimize the large cell to cell variability in response. The K^+ current elicited in response to a particular dose of TRH was normalized to the response obtained upon subsequent application of 50 nM-TRH to the same cell 6 min later (Fig. 7). Upon successive applications of 50 nM-TRH to the same cell, the second response generally tends to be around 30 % smaller. This apparent desensitization is due in part to incomplete recovery from the first response since successive applications at shorter intervals cause the second response to be even smaller. The decline,

however, may be aggravated by cell deterioration during the interval between tests. Application of 0·5 nM-TRH has no effect and the minimal detectable dose is 1 nM. A near maximal effect is achieved with 50 nM. The concentration needed to produce a half-maximal effect (EC_{50}) is approximately 7 nM.

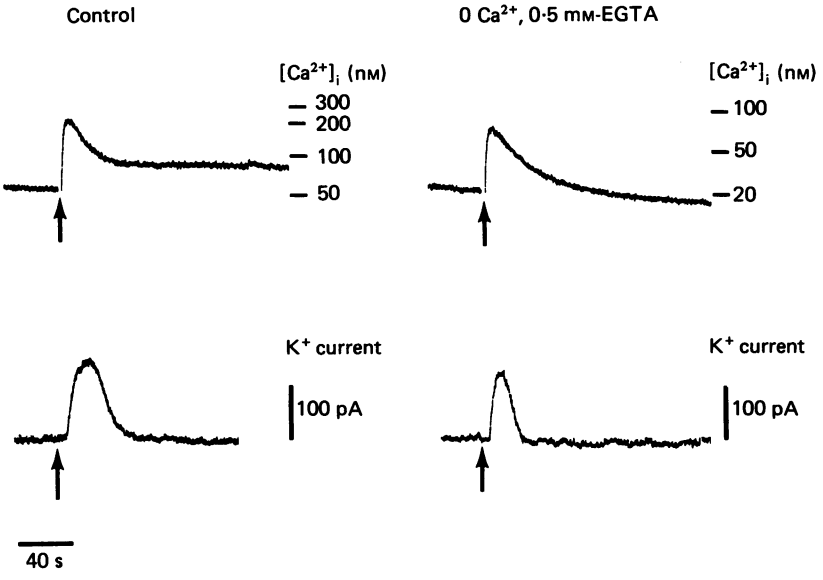

Fig. 8. Comparison of the time course of the TRH-induced change in $[Ca^{2+}]_i$ and K^+ current. The upper traces represent measurements of the intracellular free Ca^{2+} concentration of GH3 cells in suspension. The fluorescence output was monitored for quin-2-loaded cells suspended in normal saline solution (control) or in Ca^{2+}-free saline solution containing 0·5 mM-EGTA. At the time indicated by the arrow, the measurement was interrupted so that 10 μl of a concentrated solution of TRH could be added to a final concentration of 50 nM. The lower responses are current traces illustrative of the TRH-induced K^+ current measured in individual GH3 cells under voltage clamp. The holding potential was −45 mV. At the time indicated by the arrow 50 nM-TRH in normal saline solution (control) or in a Ca^{2+}-free, 0·5 mM-EGTA saline solution was continuously applied to the cell. In the latter case superfusion with Ca^{2+}-free saline was begun approximately 1 min before application of 50 nM-TRH in Ca^{2+}-free saline solution. The current traces were obtained from different cells. The time calibration is identical for all the traces shown in this Figure. In general, the time course of the response to continuous application of TRH (this Figure) was not different from the response to a 5 s application (Figs. 5 and 7).

Measurements of free cytosolic Ca^{2+} levels

Comparison of TRH-induced changes in intracellular Ca^{2+} levels and K^+ current. It has been shown by others that TRH induces a rise in cytosolic free Ca^{2+} levels (Gershengorn & Thaw, 1983, 1985; Albert & Tashjian, 1984*a*). Measurements of intracellular Ca^{2+} levels were made at room temperature with solutions identical to those used in the electrophysiological studies to examine more closely the time-dependence and dose–response relation of the TRH-induced rise in $[Ca^{2+}]_i$ as compared to the TRH-induced K^+ current (Fig. 8). These measurements were made on cells in suspension that were loaded intracellularly with quin-2, a fluorescent

indicator of Ca^{2+}. The basal $[Ca^{2+}]_i$ concentration is 74 ± 3 nM (mean $\pm$ S.E. of mean, $n = 53$). Addition of 50 nM-TRH evokes a rapid rise to a peak level (222 ± 12 nM, $n = 12$) that subsequently decays to a plateau level (109 ± 9 nM). Cells that are suspended in a Ca^{2+}-free, 0·5 mM-EGTA saline solution, have a lower basal level

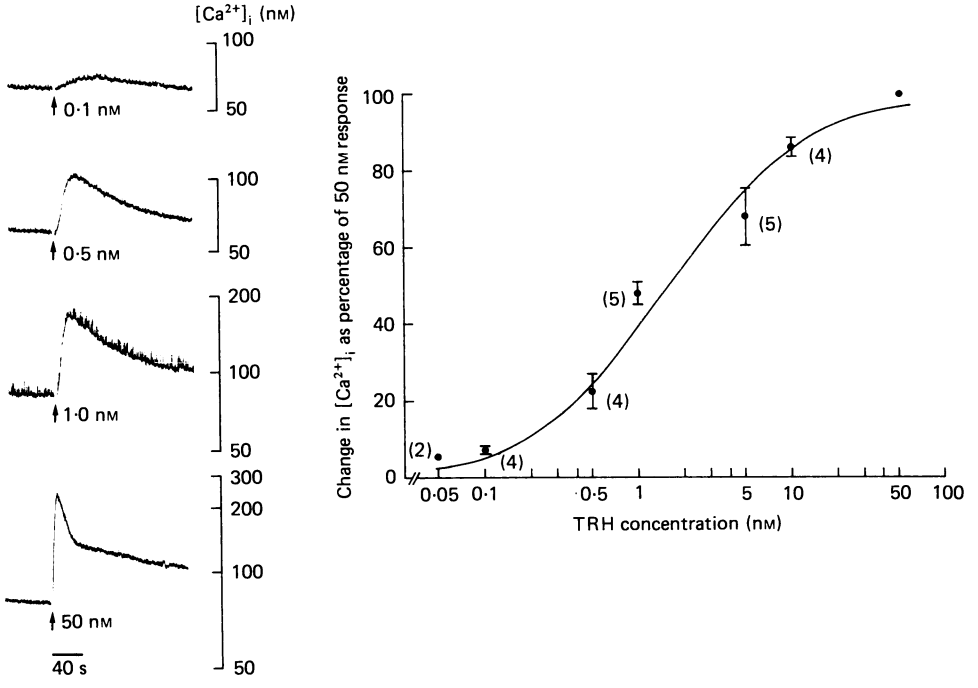

Fig. 9 Changes in $[Ca^{2+}]_i$ as a function of TRH concentration. Quin-2 fluorescent responses were obtained at room temperature in normal saline solution. Responses obtained upon addition of different concentrations of TRH are shown at the left. The loading with quin-2 and calibration of $[Ca^{2+}]_i$ were performed separately for each response. However, each of the illustrated responses was obtained on the same day from the same initial pool of unloaded cells. The dose–response curve contains responses obtained from individual loadings but from seven different pools of cells. The change in peak intracellular Ca^{2+} level after subtraction of basal level is plotted as a percentage of the response elicited by 50 nM-TRH. This percentage change is plotted against the log of the TRH concentration. Each point represents the mean $\pm$ S.E. of mean (n). The smooth curve represents the theoretical relation expected for a response that is proportional to receptor occupancy with an EC_{50} of 1·7 nM.

(42 ± 13 nM, $n = 3$). Addition of TRH continues to evoke an attenuated but rapid initial rise (93 ± 23 nM, $n = 3$). However, the plateau phase is absent since $[Ca^{2+}]_i$ subsequently declines to a level (33 ± 11 nM) that is slightly below the initial basal level. Thus, in agreement with the observations of others (Gershengorn & Thaw, 1983; Alberts & Tashjian, 1984*a*,*b*), there is an initial rapid increase in $[Ca^{2+}]_i$ that is due in part to mobilization from intracellular stores and a plateau phase that is dependent on extracellular Ca^{2+}. While extracellular Ca^{2+} may also contribute to part of the early response, the major part of the decrease in the peak is probably due to rapid depletion of intracellular stores of Ca^{2+} which occurs when GH3 cells are incubated in Ca^{2+}-free saline solution (Gershengorn, Geras, Purello & Rebecchi, 1984).

The time course of TRH-induced changes in $[Ca^{2+}]_i$ was compared with the changes in TRH-induced K^+ current (Fig. 8). The electrical response was monitored while voltage clamping the cell at a holding potential of −45 mV. Although there is considerable cell to cell variability in the time course and amplitude of the electrical responses, in general the rapid onset and duration (20–90 s) of the TRH-induced K^+ current is similar to the time course of the rapid peak increase in $[Ca^{2+}]_i$. In addition, the TRH-induced K^+current is similar in time course when cells are exposed to Ca^{2+}-free, 0·5 mM-EGTA for 1 min before and during TRH application. This is consistent with the interpretation that the TRH-induced Ca^{2+}-activated K^+ current is caused by mobilization of Ca^{2+} from intracellular stores.

When extracellular Ca^{2+} is present, no plateau phase in the TRH-activated K^+ current is observed. This result is consistent with the observation of others (Albert & Tashjian 1984*a*,*b*; Gershengorn & Thaw, 1985) that the plateau phase of intracellular Ca^{2+} occurs largely via Ca^{2+} influx through voltage-dependent Ca^{2+} channels. Under the voltage-clamp conditions activation of Ca^{2+} channels is unlikely to occur.

Dose-response relation of the TRH-induced elevation of intracellular Ca^{2+}. The log-dose–response curve for the effect of TRH on cytosolic free Ca^{2+} levels is shown in Fig. 9 along with representative responses. In the dose–response curve, the peak increase in $[Ca^{2+}]_i$ after subtraction of the basal level is expressed as a percentage of the response obtained by 50 nM-TRH in that same experiment. A near maximal response is obtained by 50 nM-TRH and the minimal detectable concentration is 0·05 nM. The EC_{50} is 1·7 nM-TRH. The levels of peak intracellular Ca^{2+} induced by 0·5 and 1 nM-TRH were 101 ± 12 nM ($n = 4$) and 152 ± 18 nM ($n = 5$), respectively.

DISCUSSION

A TRH-induced increase in membrane conductance in GH3 cells was examined using both constant-current and voltage-clamp techniques. As initially suggested by Ozawa & Kimura (1979), the TRH-induced conductance is K^+ selective and the reversal potential (−78 mV) is very close to the reversal potential that was determined from tail-current measurements of the Ca^{2+}-activated K^+ currents in GH3 cells (Ritchie, 1987).

The TRH-induced increase in K^+ conductance is not dependent on extracellular Ca^{2+}, nor is it inhibited by Ca^{2+}-channel blockers (0·5 mM-Cd^{2+}). The conductance increase, however, is abolished by introducing a Ca^{2+} chelator (20 mM-CDTA) into the cell, a treatment which completely abolishes the Ca^{2+}-activated K^+ current (Ritchie, 1987). The TRH-induced K^+ current is sensitive to inhibition by 30 mM-TEA and 200 nM-apamin, inhibitors of two pharmacologically distinct Ca^{2+}-activated K^+ currents in GH3 cells (Ritchie, 1985, 1987). Taken together, these data indicate that the TRH-induced increase in K^+ conductance is mediated by two distinct Ca^{2+}-activated K^+ conductances that do not depend on Ca^{2+} influx for activation. The present results are consistent with a recent report (Dubinsky & Oxford, 1985) which shows that application of TRH to intact GH3 cells increases the opening frequency of two different-amplitude (4 and 20 pA) channels in cell-attached, depolarized patches of membrane. The Ca^{2+} dependence of the smaller-amplitude channel was not determined; however, the larger-amplitude channel appears to be a Ca^{2+}-activated K^+ channel.

Measurements of changes in intracellular Ca^{2+}, in agreement with the observations of others (Gershengorn & Thaw, 1983, 1985; Schlegel & Wollheim, 1984; Albert & Tashjian, 1984*a*, *b*; Drummond, 1985), show that TRH induces an initial peak elevation that is due to mobilization of intracellular stores and a longer-lasting plateau phase that is dependent on Ca^{2+} influx. The plateau phase of the increase in $[Ca^{2+}]_i$ appears to be due to influx of Ca^{2+} through voltage-dependent Ca^{2+} channels (Albert & Tashjian, 1984*b*; Gershengorn & Thaw, 1985). The TRH-induced Ca^{2+}-activated K^+ current recorded under voltage-clamp conditions shows a time course that is similar to the TRH-induced initial peak increase in cytosolic free Ca^{2+} levels and is consistent with the notion that the K^+ current is activated by Ca^{2+} that is released from an intracellular store. Under voltage-clamp conditions at a -45 mV holding potential, activation of voltage-dependent Ca^{2+} channels is not expected to occur, and a plateau phase in the TRH-induced K^+ current is not observed.

TRH elevates intracellular Ca^{2+} with an EC_{50} of 1·7 nM. This is the same as the EC_{50} obtained by Albert & Tashjian (1984*a*) and is also similar to the EC_{50} for stimulation of prolactin secretion (Hinkle & Tashjian, 1973; Dannies & Tashjian, 1976). Dose–response measurements of the TRH-induced outward K^+ current revealed no effect at 0·5 nM, a small effect at 1 nM, and EC_{50} of approximately 7 nM. Lack of effect by 0·5 nM-TRH is surprising since 0·5 nM increases $[Ca^{2+}]_i$ from the basal level of 74 nM to a peak value of 101 ± 24 nM ($n = 4$). There are several possible explanations for this lack of correspondence. (1) Since quin-2 senses the average $[Ca^{2+}]_i$ while K^+ channels detect Ca^{2+} at the periphery, diffusion and binding may limit access of released Ca^{2+} to peripheral sites. (2) The introduction of the patch electrode into the cell probably perturbs the level of $[Ca^{2+}]_i$ at rest and during stimulation by TRH. (3) The higher EC_{50} for the electrical response may indicate that a threshold level of $[Ca^{2+}]_i$ that is greater than 100 nM is needed to activate the K^+ channels at the -45 mV holding potential that was used to observe the effects. We are currently attempting to resolve these inconsistencies by measuring the voltage and Ca^{2+} sensitivities of the Ca^{2+}-activated K^+ channels in inside-out patches of GH3 membranes.

The TRH-induced initial peak increase and the later plateau in $[Ca^{2+}]_i$ correlate in time with a biphasic pattern of stimulation of prolactin secretion (Albert & Tashjian, 1984*b*; Kolesnik & Gershengorn, 1985). The plateau phase of the increase in cytosolic Ca^{2+} also correlates in time with a delayed increase in action potential frequency that has been observed by others (Kidokoro, 1975; Ozawa & Kimura, 1979; Dufy *et al.* 1979; Dubinsky & Oxford, 1985). In the present study the delayed increase in action potential frequency was rarely observed even though the measurements of cytosolic free Ca^{2+} levels reveal the plateau phase of the rise in cytosolic Ca^{2+}. It is possible that introduction of the patch recording electrode into the cell resulted in diffusional loss of cytoplasmic contents that are necessary for mediating the TRH-induced facilitation of action potential frequency (see Dufy, MacDermott & Barker, 1985). Others have shown that TRH causes inhibition of a rapidly inactivating, voltage-dependent K^+ current (Sand, Haug & Gautvik, 1980; Dufy & Barker, 1983; Dubinsky & Oxford, 1984, 1985) which may be the underlying cause for the facilitation of Ca^{2+} action potentials.

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